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14. ABSTRACT The overall objective is to define the interaction between Notch and VEGFR-3 signaling in breast cancer. We are examining a role for Notch in breast tumor vessels and attempting to block Notch and VEGFR-3 activity in breast tumors grown in mice. We proposed two aims: 1) studies of Notch/Dll4 function in murine mammary tumorigenesis and 2) studies of the inhibitory effects of a Notch antagonist (Notch decoy) in a murine mammary tumor model. In aim 1, to study the role for notch in murine mammary tumorigenesis, progress has been made in developing two new transgenic lines that will allow for conditional activation or inactivation of Notch specifically within the endothelium. The first mouse line, EF1-Notch1IC can be manipulated in a conditional fashion, as demonstrated by lethality if activated in embryonic endothelium. The second mouse line, EF1-Notch1ECD/Fc, has been generated and is being further tested. We have carried out experiments to demonstrate that breast tumor xenograft growth is inhibited by the Notch decoy, an antagonist made up of the Notch1 extracellular domain fused to the Fc protein, Notch1ECD/Fc. This block appears to be a result of reduced tumor angiogenesis. This strategy, now shown to inhibit mammary tumor growth in our mouse models, is proving to be a promising area for therapeutic intervention in breast cancer.					
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INTRODUCTION

Expression studies have shown that the angiogenic/lymphangiogenic factor VEGFR-3, its ligand VEGF-C and the Notch ligand, Dll4 are up-regulated in the invading blood and lymphatic vessels in human breast cancer (1, 2). Using mouse models, Notch signaling has been found to be essential for angiogenesis to progress to completion in the developing embryo (3). However, the exact mechanism(s) by which Notch signaling regulates angiogenesis and/or lymphangiogenesis is not well understood. It is known that Notch modulates cell-fate decisions by regulating the expression of tissue specific genes. As many angiogenic regulators have been identified, we have focused on defining the interactions between Notch and that of known angiogenic regulators. Using quantitative RT-PCR analysis, we found that VEGFR-3 was induced in response to an activated form of Notch4 (Notch4/int-3) in three human primary endothelial cells (HUVEC-umbilical vein, HUAEC-umbilical artery, and HMVEC-dermal microvascular). Taken together these data suggested a relationship between Notch signaling and VEGFR-3. Thus, we ***hypothesized*** that Notch may regulate blood and/or lymphatic vessel development via its induction of VEGFR-3 during physiological angiogenesis, as well as pathological angiogenesis and/or lymphangiogenesis in breast cancer. The ***overall objective*** of this proposal is to define the interaction between Notch and VEGFR-3 signaling in breast cancer.

Our original study design was as follows: We will investigate the relationship between Notch and VEGFR-3 signaling in gain-of-function and loss-of-function mouse models. We have developed two mouse models in which Notch signaling can be specifically induced in the vascular endothelium. Murine mammary tumor cells will be implanted into these mice and the affect(s) of Notch signaling on tumor angiogenesis/lymphangiogenesis determined. Expression of Dll4 and VEGFR-3 and its ligands, VEGF-C and VEGF-D will also be monitored. We will also determine if blocking endogenous Notch signaling will perturb breast tumor angiogenesis/lymphangiogenesis. We have generated Notch antagonists, the Notch decoys, which encode the extracellular domains of the four Notch proteins but not the signaling domain. Human mammary tumor cells lines will be generated that express the different Notch antagonists and xenografted into immuno-compromised mice. The affects of the Notch1 antagonist on tumor angiogenesis and/or lymphangiogenesis will be examined, as well as the affects on Dll4 and VEGFR-3 expression.

In year two we made progress on three of our designated tasks. In **Task 1** (Months 1-3) we set out to optimize conditions for tamoxifen treatment of mice to conditionally activate Notch. We have made progress in establishing that Notch1C can be conditionally activated using our altered strategy to develop a new transgenic Notch1C line and a transgenic Notch decoy (inhibitor) line and our progress will be described. In **Task 2** (Months 4-24) we set out to induce Notch activity in adult mice and determine the consequences for breast tumor cell growth. The mice needed for this task, EF1-Notch1C and EF1-Notch1ECD/Fc are now generated and validated. In **Task 4** (Months 6-36) analysis of VEGFR-3 expression in tumors expressing Notch antagonists. An initial goal in this task was to develop breast cancer cell lines over-expressing Notch1C or Notch decoy in order to manipulate Notch activity in the cancer cell. Ultimately, we wanted to create line that secrete decoy to assess the affects on tumor cell/endothelial interaction. What we found was that Notch activity affected the cancer cell. Activation of Notch suppressed breast cancer cell growth and expression of Notch decoy promoted growth. This result was unexpected and will be discussed in the body, below. However, we now have worked with a mouse mammary tumor line, Mm5MT, which is not affected by the Notch decoy when analyzed in vitor. When analyzing the consequences of Notch decoy expression in xenografted breast cancer cells, we found that tumor growth was indeed inhibited by the Notch decoy.

BODY

Specific Aims 1: Notch and Dll4 function in murine mammary tumorigenesis.

Task 1 (Months 1-3) Optimizing conditions for TM treatment of N1ICD and Dll4 double transgenic mice

Experiments using both gain-of-function and loss-of-function mouse breast tumor models will aid our understanding of the role of Notch in tumor angiogenesis. The receptors for Dll4, Notch1 and Notch4 are endogenously expressed in endothelial cells (4, 5). Notch4 activation in embryonic endothelium causes vascular remodeling defects in murine embryos (6). Notch4 nullizygous mice are viable and fertile (7). However, we noticed that the adults have vascular patterning defects. Notch4 is primarily expressed within the vascular endothelium during development and this expression is maintained in the adult (8). In our original proposal, we pursued a system to conditionally express N1ICD, an activated form of Notch1, in the adult vasculature. We have generated mice doubly transgenic for a Flk1-Cre-ERT transgene and an N1ICD CAG-CAT transgene in the C57BL/6J background. The Flk1-Cre-ERT transgene encodes the Flk1 (VEGFR-2) regulatory region upstream of a Cre-estrogen receptor fusion gene. In the adult mouse, Flk1 expression is restricted the vascular endothelium. Thus, this transgene limits the expression of the Cre-ERT fusion protein to the vascular endothelial cells. The Cre-ERT fusion protein has a tamoxifen(TM)-responsive estrogen receptor binding component and is latent until TM is administered. The CAG-CAT transgene contains a non-functional gene (CAT) with its associated stop codon, flanked by lox P sites followed by either N1ICD or Dll4 gene. In the absence of Cre expression, the CAT gene is expressed. When the CAT gene is excised by Cre, the N1ICD or Dll4 gene is placed behind the CAG promoter and expressed. The CAG promoter consists of a CMV enhancer-chicken b-actin hybrid

protomoter that is strongly expressed in endothelial cells. Thus, when treated with tamoxifen these double transgenic mice activate Notch signaling specifically within the vascular endothelium (data not shown).

Figure 1A

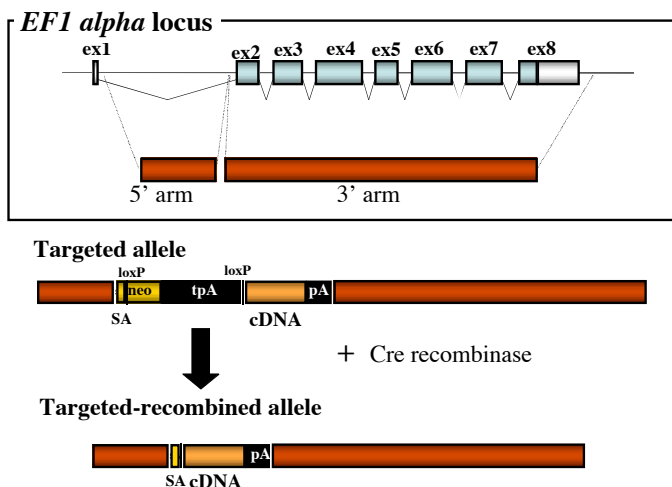
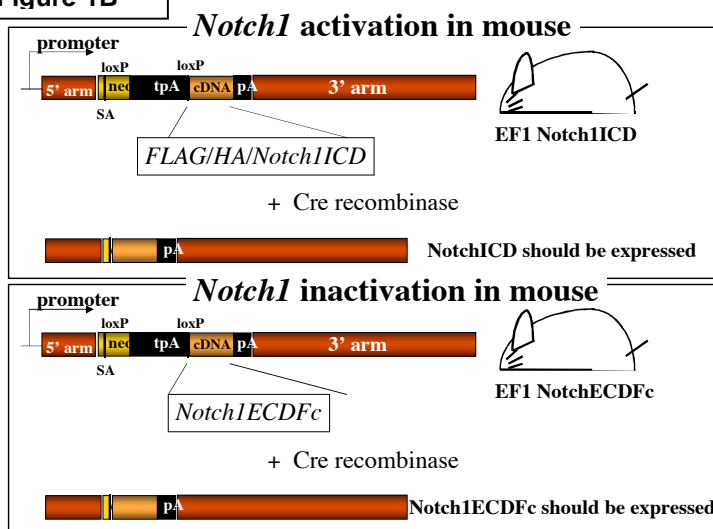


Figure 1B



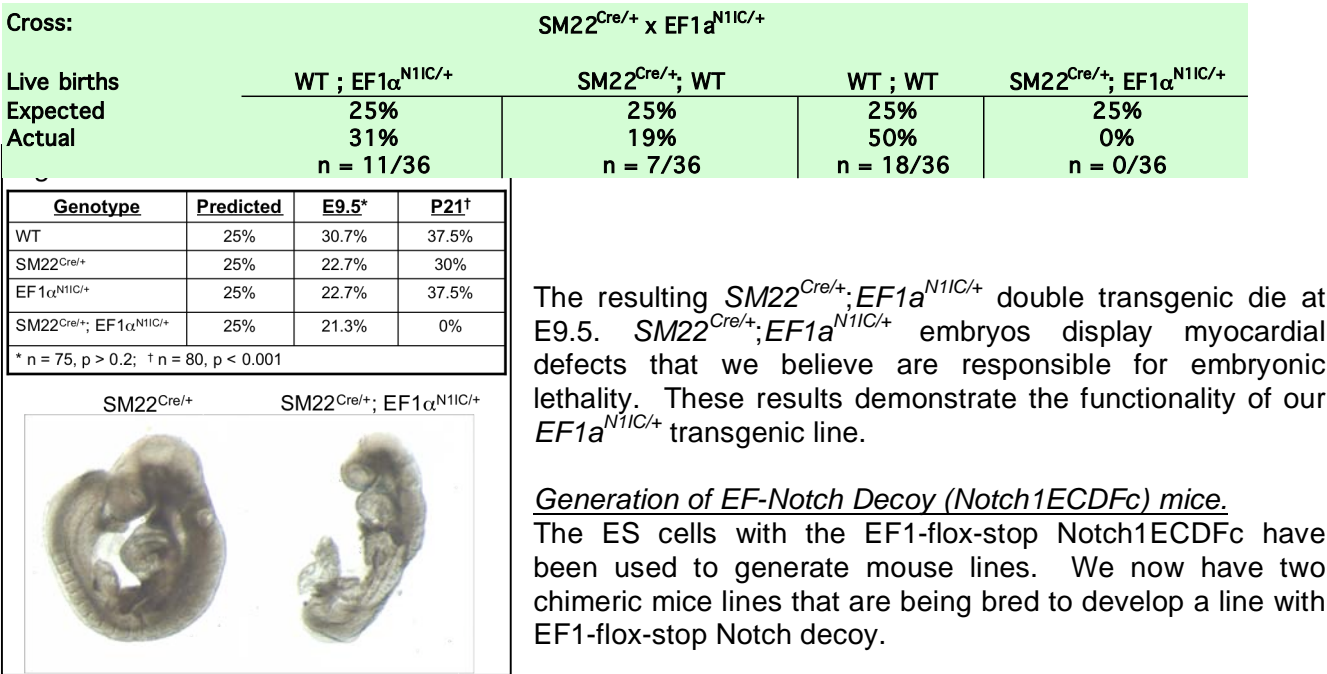
In this new strategy, we also include a mouse line designed to inactivate Notch signaling in tumor endothelium that incorporates the Notch decoy. Although these approaches were not originally

proposed, we feel that inclusion enhances our ability to understand Notch function in tumor endothelium by either activating or inactivating Notch in tumor vessels.

The EF1-flox-Notch constructs use the strong promoter from the eukaryotic Elongation Factor 1 (EF1) gene to drive expression (Figure 1A). Using homologous recombination, we have inserted a flox-stop-Notch1IC construct and a flox-stop-NotchECDFc (Notch decoy) construct into the EF1 locus (Figure 1B). The proper targeting of ES cells has been confirmed and a mouse has already been generated for expression of Notch1IC (EF1-flox-stop-Notch1IC). This will be used in the same fashion as CAG-CAT-Notch1IC. To date, we have crossed EF1-flox-stop-Notch1IC mice with Rosa-cre mice and this leads to embryonic lethality, as expected. The mice have also been crossed to the endothelial specific Flk1-cre mouse and this also leads to embryonic lethality. Thus, abnormal activation of Notch, either globally or in an endothelial specific fashion, is not compatible with proper embryonic development.

In order to further test the EF1-Notch1IC mice, we crossed to a mouse line that expresses cre recombinase in vascular smooth muscle cells, SM22cre. This cross did not produce viable doubly transgenic offspring, SM22cre; EF1-N1IC (Figure 2). The resulting $SM22^{Cre/+};EF1\alpha^{N1IC/+}$ double transgenic die at E9.5. $SM22^{Cre/+};EF1\alpha^{N1IC/+}$ embryos display myocardial defects that we believe are responsible for embryonic lethality. These results demonstrate the functionality of our $EF1\alpha^{N1IC/+}$ transgenic line. Thus, expression of N1IC via either an endothelial- or vascular smooth muscle-specific cre leads to lethality, validating the use of this coniditional line for the study of Notch in tumor vasculature.

Figure 2. Result of cross of SM22 cre-recombinase mice and EF1-Notch1IC



Specific Aims 2: Analysis of Notch antagonists in a murine mammary tumor model

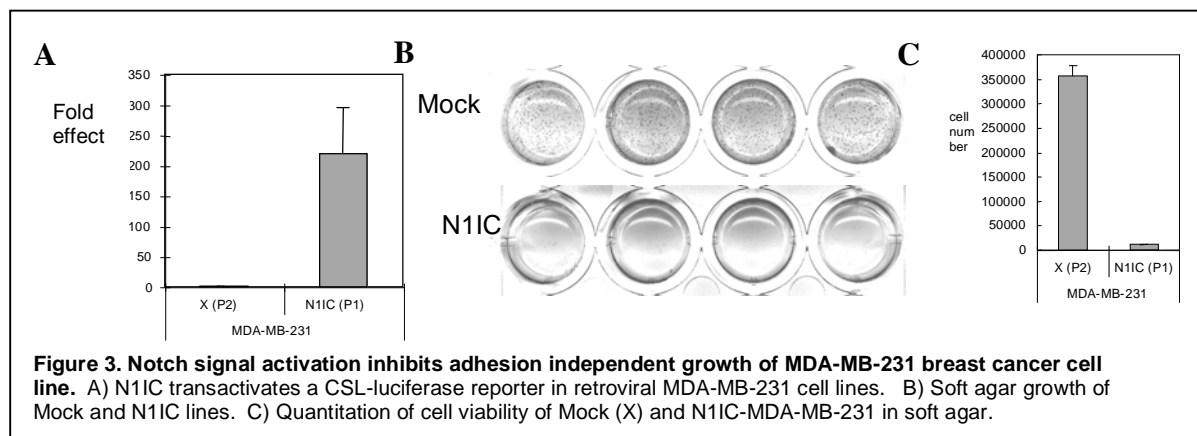
Task 4 (Months 6-36) Analysis of VEGFR-3 expression in tumors expressing Notch antagonist

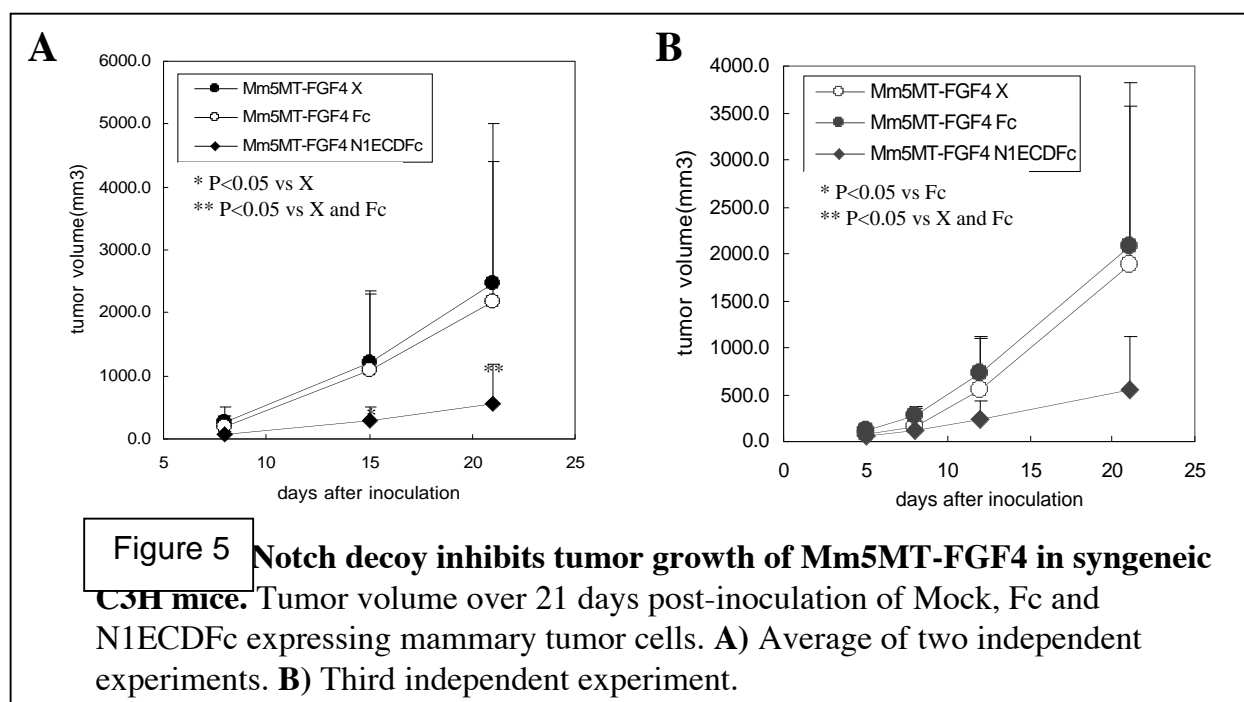
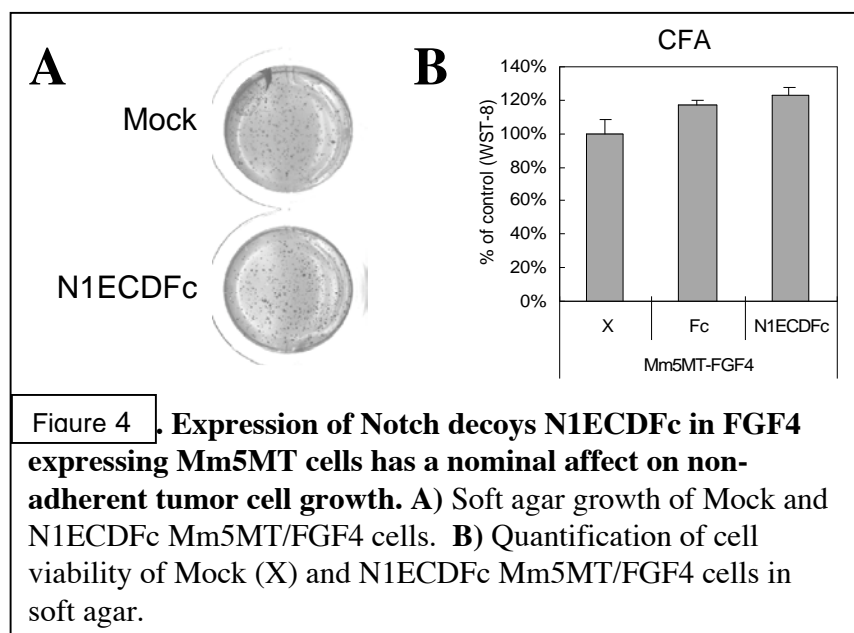
In previous studies of murine xenografts of human mammary tumor cells, Dll4 expression was found to be enhanced within the tumor vessels (2). Moreover, ectopic expression of the VEGFR-3 ligand, VEGF-C, in mammary xenografts induced lymphangiogenesis and nodal spread of the breast

cancer cell (9, 10). Taken together with the observation that Notch signaling induces VEGFR-3 expression in cultured endothelial cells, we want to test whether blocking Notch activity suppressed tumor growth and pathological angiogenesis/lymphangiogenesis. We have generated Notch antagonists that are composed of the signal peptide and EGF-like repeats of Notch1 and Notch2 fused in frame with Fc fragment of human IgG. Using an *in vitro* co-culture assay, we have found that the Notch antagonists N1ECDFc and N2ECDFc perturb ligand-activated signaling of Notch1, Notch2 and Notch4 (data not shown). Thus, we proposed to generate mammary tumor cell lines expressing these Notch antagonists and transplant them into immunocompromised mice.

Analysis of VEGFR-3 expression in tumors expressing Notch antagonists. In previous studies of murine xenografts of human mammary tumor cells, Dll4 expression was found to be enhanced within the tumor vessels Delta4, an endothelial specific notch ligand expressed at sites of physiological and tumor angiogenesis (2). Moreover, ectopic expression of the VEGFR-3 ligand, VEGF-C, in mammary xenografts induced lymphangiogenesis and nodal spread of the breast cancer cells (9). VEGF-C-mediated lymphangiogenesis promoted tumor metastasis and VEGF-C induced lymphangiogenesis is associated with lymph node metastasis in orthotopic MCF-7 tumors (10). Taken together with the observation that Notch signaling induces VEGFR-3 expression in cultured endothelial cells, we want to test whether blocking Notch activity suppressed tumor growth and pathological angiogenesis/lymphangiogenesis. We have generated Notch antagonists that are composed of the signal peptide and EGF-like repeats of Notch1 and Notch2 fused in frame with Fc fragment of human IgG. Using an *in vitro* co-culture assay, we have found that the Notch antagonists N1ECDFc and N2ECDFc perturb ligand-activated signaling of Notch1, Notch2 and Notch4 (data not shown). Thus, we proposed to generate mammary tumor cells expressing these Notch antagonists and transplant them into mice.

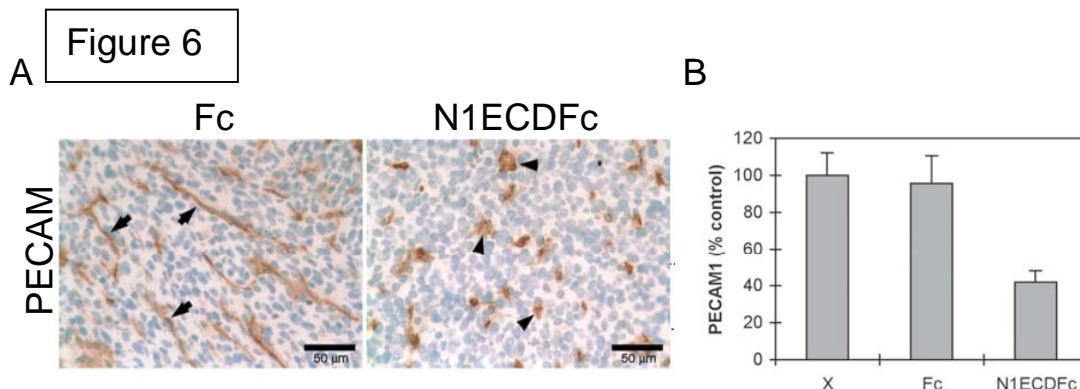
Our expression studies of human mammary tissue demonstrated that Notch1, Notch4 and Dll4 were expressed in the normal and malignant ductal epithelial cells. This suggested to us that Notch signaling functions in normal as well as malignant ductal epithelium. Therefore, we decided to determine the affects of Notch signal activation on growth and tumorigenicity of human MDA-MB-231 breast cancer cell line and FGF expressing mouse Mm5MT mammary tumor cell line. MDA-MB-231 and FGF expressing Mm5MT cells were retrovirally transduced with empty virus or viruses expressing activated forms of Notch1 (N1IC) or Notch4 (N4/int-3). Cell populations were selected in hygromycin. Consistent with Notch signaling being activated, cell populations expressing N1IC or N4/int-3 transactivated luciferase reporters encoding 6 Notch/CSL binding sites relative to the mock infected controls (Figure 3A). Next, we determined the ability of the mock and Notch expressing mammary tumor populations to form colonies in soft agar (Figure 3B). Notch signal activation inhibited adhesion independent growth of the transformed mammary tumor lines. In MDA-MB-231, Notch1 signaling suppressed cell growth greater than 20 fold relative to the mock-infected cells (Figure 3C). The inhibition of cell growth was also observed in monolayer cultures and thus, they could not be subcutaneously transplanted in immunocompromised mice.





N1ECDFc suppresses angiogenesis in a mouse model of breast cancer Next, we determined the affect of expressing the Notch antagonist, N1ECDFc on the tumorigenicity of mammary tumor cells. FGF-expressing Mm5MT cells were retrovirally infected with empty virus, Fc (control) or N1ECDFc expressing viruses and cell populations generated by hygromycin selection. In soft agar assays, expression of the Notch antagonists had a nominal affect on soft agar growth as determined by WST-We evaluated our Notch antagonist, N1ECDFc, in a mouse model of breast cancer to determine its effects on tumor growth and angiogenesis. In this tumor model, FGF4-expressing Mm5MT (FGF4-Mm5MT) are tumorigenic when subcutaneously implanted in syngeneic mice (data not show). In this tumor model, angiogenesis is promoted by hypoxia-driven VEGF-A expression. We infected FGF4-Mm5MT with empty vector lentivirus, or lentiviruses encoding either human Fc or N1ECDFc. Tumor lines were implanted subcutaneously and allowed to grow for 21 days. Two independent trials were monitored for tumor growth for 3 weeks (Figure 5). In the two trials,

expression of N1ECDFc significantly inhibited tumor growth. Mice were sacrificed, tumors removed and PECAM staining performed to visualize blood vessel density and morphology (Fig. 6A). Blood vessels were stunted and disorganized in the FGF4-Mm5MT expressing N1ECDFc, as compared to Fc control. The blood vessel density was reduced nearly 60% in the N1ECDFc-expressing tumors relative to the mock (X) and Fc-expressing FGF4-Mm5MT (Fig. 6B).



Notch1 and Notch4 are co-expressed in the breast cancer lymphatic endothelium.

We observed that Notch1, Notch4, Dll4 and VEGFR-3 were all expressed in the vasculature of human breast carcinoma (data not shown). We also observed that Jagged1 is expressed with the vasculature of normal breast tissue (data not shown). Whether these proteins are expressed in both the blood and lymphatic endothelium remains to be determined. To discriminate between the blood and lymphatic vessels, we performed immunohistochemistry on human micropapillary breast carcinomas using antibodies against CD34, as a marker for blood endothelium and LYVE-1 and podoplanin as markers for lymphatic endothelium (Fig. 7).

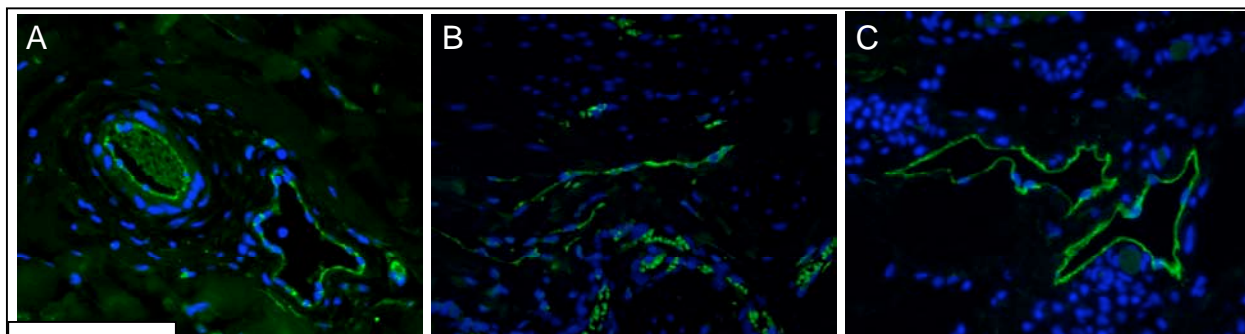


Figure 7

Expression of blood and lymphatic endothelial cell markers in micropapillary breast carcinomas. A) CD34 expression in the blood endothelium. Expression of the lymphatic markers B) LYVE-1 and C) podoplanin.

We then performed double immunohistochemistry with antibodies against LYVE-1 and Notch1 or Notch4 (Fig. 8). We found that both Notch1 and Notch4 are expressed in the lymphatic endothelium of human micropapillary breast carcinomas. Notch1 and Notch4 were co-expressed with LYVE-1 in the lymphatic endothelial cell bodies. Interestingly, Notch1 and Notch4 proteins were also detected in the nuclei of the lymphatic endothelial cells suggesting that Notch signaling was activated in these cells (Fig. 7 G & H). To further validate this observation, we co-immunostained the micropapillary breast carcinoma tissue with Notch1Val, an antibody that only recognizes the activated Notch1 peptide and the lymphatic marker, podoplanin (Fig. 9). Activated Notch1 peptide was detected in a subset of the tumor lymphatic endothelium.

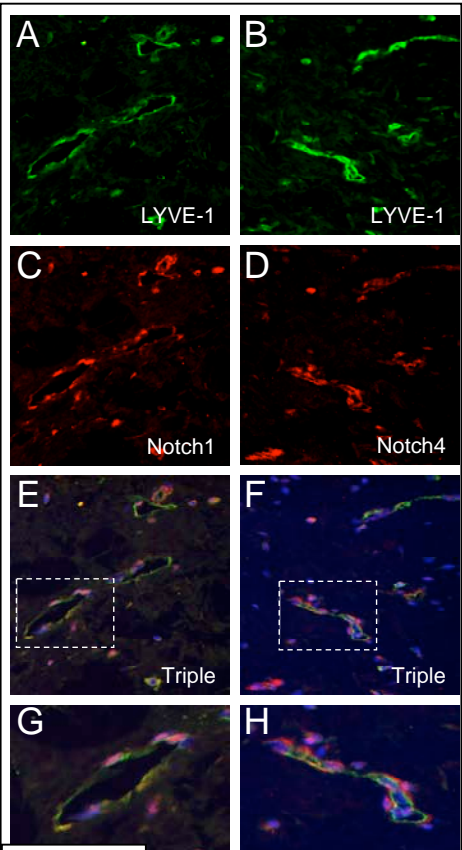


Figure 8 Notch1 and Notch4 are expressed in the lymphatics of micropapillary breast carcinomas. A, B) LYVE-1, C) Notch1, D) Notch4 double immunohistochemistry of breast tumor tissue. E) Notch1 and LYVE-1 co-expression in the lymphatic vessel. F) Notch4 and LYVE-1 co-expression in the lymphatic vessel. G, H) Magnified view of lymphatic vessels from panels E and F, respectively.

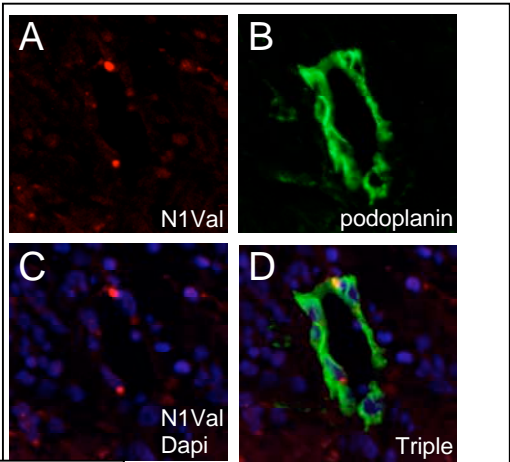
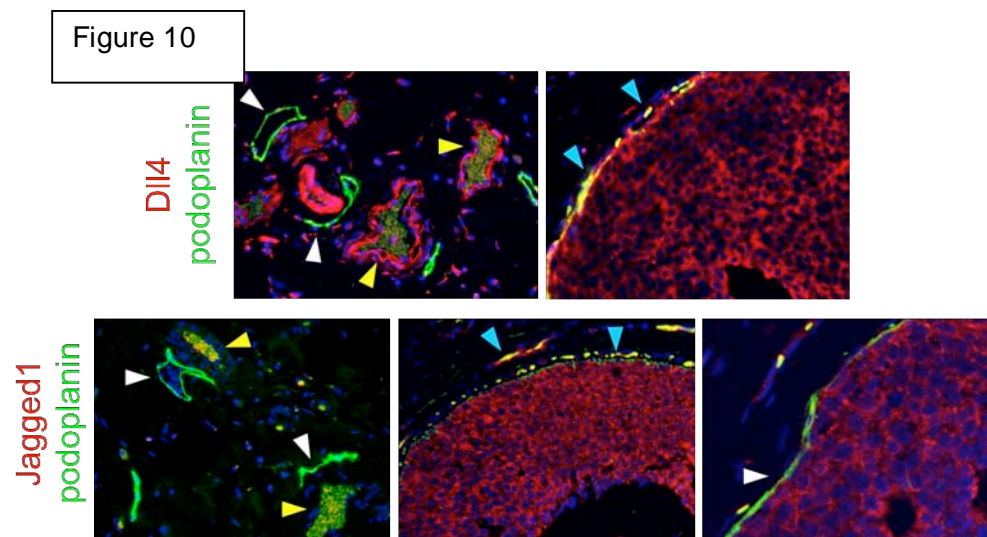


Figure 9 Notch1 is activated in a lymphatic endothelium of micropapillary breast carcinomas. A) Notch1 Val, and B) Podoplanin double immunohistochemistry of breast tumor tissue. C) DAPI and Notch1 Val image demonstrating perinuclear and nuclear expression of activated Notch1. D) Triple stain, Notch1 Val, podoplanin and DAPI.

Dll4 and Jagged1 are differentially expressed in human breast cancer lymphatic vessels. Since Notch1 and Notch4 were expressed in breast cancer extratumoral lymphatics, we examined the expression of the Notch ligands, Dll4 and Jagged1. Human ductal carcinoma in situ (DCIS) were co-stained with either Dll4 or Jagged1 (red), and podoplanin (green) antibodies. In the uninvolved tissue, Dll4 expression was restricted to podoplanin-negative blood vessels (yellow arrows) as evidence of the red blood cells (Fig. 10 left upper panel). In contrast, Dll4 and podoplanin expression was coincident (blue arrows) in the lymphatic vessels adjacent the DCIS (Fig. 10 right upper panel). Jagged1 was expressed in neither blood (yellow arrows), nor lymphatic vessels (white arrows) in the uninvolved tissue (Fig. 10 left lower panel). In the lymphatics surrounding the DCIS, we found that Jagged1 was co-expressed with podoplanin in some cases (blue arrows; Fig. 10 middle lower panel) and not in others (white arrow; Fig. 10 right lower panel). The expression of Dll4 and Jagged1 in the lymphatic vessels encircling DCIS suggest that Notch signaling may have a role in pathological lymphangiogenesis associated with breast cancer.



KEY RESEARCH ACCOMPLISHMENTS

- Developed a EF-1-flox-Notch1C mouse line that expresses an activated form of Notch1 with within the murine vasculature when crossed with either an endothelial specific driven Cre or a vascular smooth muscle-specific cre.
- Generated EF-1-flox-Notch decoy mice for conditional loss-of-function Notch mice.
- Found that the expression of activated Notch constructs or Notch antagonists altered the growth of murine and human mammary tumor cell lines in soft agar.
- Found that the expression of activated Notch constructs inhibited the growth of murine and human mammary tumor cell lines in soft agar.
- Demonstrated that the Notch1 antagonist inhibited tumor growth of subcutaneous xenografts of mouse mammary tumor cells.
- Demonstrated that Notch1, Notch4, Delta-4, and Jagged-1 are expressed and most likely active in the lymphatic endothelium of micropapillary breast carcinomas.

REPORTABLE OUTCOMES (PUBLICATIONS/ABSTRACTS)

Publications:

Vorontchikhina, M.A., Zimmerman, R.C., Shawber, C.J., Tang, H., and **Kitajewski, J.** (2005) Unique patterns of Notch1, Notch4 and Jagged1 expression in ovarian vessels during folliculogenesis and corpus luteum formation. *Gene Expression Patterns*. (5 : 701-9)

Zeng Q, Li S, Chepeha DB, Giordano TJ, Li J, Zhang H, Polverini PJ, Nor J, **Kitajewski J**, Wang CY. (2005) Crosstalk between tumor and endothelial cells promotes tumor angiogenesis by MAPK activation of Notch signaling. *Cancer Cell* **8**:13-23.

Abstract:

Shawber, C.J., Funahashi, Y., Francisco, E., Podgrabinska, S., Kitamura, Y., Vorontchikhina, M., Shiraishi, K., Chawengsaksophak, K., Rossant, J., Accili, D., Skobe, M., and **Kitajewski, J.** (2006) *VEGFR-3, a direct endothelial cell specific target of Notch signaling in vitro and in vivo*. Molecular Mechanisms in Lymphatic Function and Disease. Gordon Research Conference. Les Diablerets, Switzerland

Shawber, C.J., Francisco, E., Feirt, N., Roe, T., Podgrabinska, S., Kitamura, Y., Shiraishi, K., Chawengsaksophak, K., Rossant, J., Accili, D., Skobe, M., and **Kitajewski, J.** *Role of Notch/VEGFR-3 in breast tumor angiogenesis and lymphangiogenesis*. (2005) Era of Hope. Department of Defense Breast Cancer Research Program Meeting. Philadelphia, PA.

Shawber, C.J., Funahashi, Y., Francisco, E., Feirt, N., Roe, T., Podgrabinska, S., Kitamura, Y., Vorontchikhina, M., Shiraishi, K., Chawengsaksophak, K., Rossant, J., Accili, D., Skobe, M., and **Kitajewski, J.** *Notch and VEGFR-3 interactions in endothelial cells and breast cancer vasculature*. (2005) Era of Hope. Department of Defense Breast Cancer Research Program Meeting. Philadelphia, PA.

Shawber, C.J., Funahashi, Y., Francisco, E., Podgrabinska, S., Kitamura, Y., Vorontchikhina, M., Shiraishi, K., Chawengsaksophak, K., Rossant, J., Accili, D., Skobe, M., and **Kitajewski, J.** (2005) *VEGFR-3, a direct endothelial cell specific target of Notch signaling in vitro and in vivo*. Vascular Cell Biology. Gordon Research Conference. Ventura, California.

Vorontchikhina, M., Shawber, C., Zimmerman, R. and **Kitajewski, J.** (2004) *Expression of Notch and Notch ligands during folliculogenesis and corpus luteum formation marks a subset of ovarian vessels*. The XIIIth International Vascular Biology Meeting. Toronto, Canada.

Shawber, C., Funahashi, Y., Francisco, E., Podgrabinska, S., Vorontchikhina, M., Shiraishi, K., Chawengsaksophak, K., Rossant, J., Skobe, M. and **Kitajewski, J.** (2004) *Identification and Characterization of Notch Regulated Genes in Blood and Lymphatic Endothelial Cells*. Molecular Mechanisms in Lymphatic Function and Disease. Gordon Research Conference, Ventura, California.

CONCLUSIONS

The proposal **objective** is to define the interaction between Notch and VEGFR-3 in breast cancer. To study the role for notch in murine mammary tumorigenesis, progress has been made in developing two new transgenic lines that will allow for conditional activation or inactivation of Notch specifically within the endothelium. These will be used to manipulate Notch activity in tumor endothelium of mice. angiogenesis. The first mouse line, EF1-Notch1IC can be manipulated in a conditional fashion, as demonstrated by lethality if activated in embryonic endothelium. The second mouse line, EF1-Notch1ECD/Fc, has been generated and is being further tested. We have carried out experiments to demonstrate that breast tumor xenograft growth is inhibited by the Notch decoy, an antagonist made up of the Notch1 extracellular domain fused to the Fc protein, Notch1ECD/Fc. This block appears to be a result of reduced tumor angiogenesis. This strategy, now shown to inhibit mammary tumor growth in our mouse models, is proving to be a promising area for therapeutic intervention in breast cancer.

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